MPMI Vol. 8, No. 4, 1995, pp. 484–[illegible]

Erwinia chrysanthemi Harpin$_{Ech}$: An Elicitor of the Hypersensitive Response that Contributes to Soft-Rot Pathogenesis

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Received 14 December 1994. Accepted 8 March 1995.

Mutants of the soft-rot pathogen *Erwinia chrysanthemi* EC16 that are deficient in the production of the pectate lyase isozymes PelABCE can elicit the hypersensitive response (HR) in tobacco leaves. The *hrpN*$_{Ech}$ gene was identified in a collection of cosmids carrying *E. chrysanthemi hrp* genes by its hybridization with the *Erwinia amylovora hrpN*$_{Ea}$ gene. *hrpN*$_{Ech}$ appears to be in a monocistronic operon, and it encodes a predicted protein of 340 amino acids that is glycine-rich, lacking in cysteine, and highly similar to HrpN$_{Ea}$ in its C-terminal half. *Escherichia coli* DH5α cells expressing *hrpN*$_{Ech}$ from the *lac* promoter of pBluescript II accumulated HrpN$_{Ech}$ in inclusion bodies. The protein was readily purified from cell lysates carrying these inclusion bodies by solubilization in 4.5 M guanidine-HCl and reprecipitation upon dialysis against dilute buffer. HrpN$_{Ech}$ suspensions elicited a typical HR in tobacco leaves, and elicitor activity was heat-stable. Tn*5-gusA1* mutations were introduced into the cloned *hrpN*$_{Ech}$ and then marker-exchanged into the genomes of *E. chrysanthemi* strains AC4150 (wild type), CUCPB5006 (Δ*pelABCE*), and CUCPB5030 (Δ*pelABCE outD*::Tn*phoA*). *hrpN*$_{Ech}$::Tn*5-gusA1* mutations in CUCPB5006 abolished the ability of the bacterium to elicit the HR in tobacco leaves unless complemented with an *hrpN*$_{Ech}$ subclone. An *hrpN*$_{Ech}$::Tn*5-gusA1* mutation also reduced the ability of AC4150 to incite infections in witloof chicory leaves, but it did not reduce the size of lesions that did develop. Purified HrpN$_{Ech}$ and *E. chrysanthemi* strains CUCPB5006 and CUCPB5030 elicited HR-like necrosis in leaves of tomato, pepper, African violet, petunia, and pelargonium, whereas *hrpN*$_{Ech}$ mutants did not. HrpN$_{Ech}$ thus appears to be the only HR elicitor produced by *E. chrysanthemi* EC16, and it contributes to the pathogenicity of the bacterium in witloof chicory.

The hypersensitive response (HR) is a rapid, localized necrosis that is associated with the active defense of plants against many pathogens (Kiraly 1980; Klement 1982). The HR elicited by bacteria is readily observed as a tissue collapse if high concentrations (≥10^7 cells per milliliter) of a limited-host-range pathogen like *Pseudomonas syringae* or *Erwinia amylovora* are infiltrated into leaves of nonhost plants (necrosis occurs only in isolated plant cells at lower levels of inoculum) (Klement 1963; Klement *et al.* 1964; Turner and Novacky 1974; Klement 1982). The capacities to elicit the HR in a nonhost and to be pathogenic in a host appear linked. As noted by Klement (1982), these pathogens also cause physiologically similar, albeit delayed, necroses in their interactions with compatible hosts. Furthermore, the ability to produce the HR or pathogenesis is dependent on a common set of genes, denoted *hrp* (Lindgren *et al.* 1986; Willis *et al.* 1991). Consequently, the HR may hold clues to both the nature of plant defense and the basis for bacterial pathogenicity.

The *hrp* genes are widespread in gram-negative plant pathogens, where they are clustered, conserved, and in some cases interchangeable (Willis *et al.* 1991; Bonas 1994). Several *hrp* genes encode components of a protein secretion pathway similar to one used by *Yersinia*, *Shigella*, and *Salmonella* spp. to secrete proteins essential in animal diseases (Van Gijsegem *et al.* 1993). In *E. amylovora*, *P. syringae*, and *P. solanacearum*, *hrp* genes have been shown to control the production and secretion of glycine-rich protein elicitors of the HR (He *et al.* 1993; Wei and Beer 1993; Arlat *et al.* 1994).

The first of these proteins was discovered in *E. amylovora* Ea321, a bacterium that causes fire blight of rosaceous plants, and was designated harpin (Wei *et al.* 1992). Mutations in the encoding *hrpN* gene revealed that harpin is required for *E. amylovora* to elicit the HR in nonhost tobacco leaves and incite disease symptoms in highly susceptible pear fruit. The *P. solanacearum* GMI1000 PopA1 protein has similar physical properties and also elicits the HR in leaves of tobacco, which is not a host of that strain (Arlat *et al.* 1994). However, *P. solanacearum popA* mutants still elicit the HR in tobacco and incite disease in tomato. Thus, the role of these glycine-rich HR elicitors can vary widely among gram-negative plant pathogens.

E. chrysanthemi is unlike the bacterial pathogens that typically elicit the HR because it has a wide host range, rapidly kills and macerates host tissues, and secretes several isozymes of the macerating enzyme pectate lyase (Pel) (Barras *et al.* 1994). Nevertheless, PelABCE$^-$ and Out$^-$ (pectic enzyme secretion pathway) mutants of *E. chrysanthemi* EC16 cause a typical HR (Bauer *et al.* 1994). Furthermore, elicitation of the HR by *E. chrysanthemi* is dependent on an *hrp* gene that is conserved in *E. amylovora* and *P. syringae* and functions in the secretion of the *E. amylovora* harpin (Wei and Beer 1993; Bauer *et al.* 1994). Mutation of this gene significantly reduces the ability of *E. chrysanthemi* to incite lesions in susceptible

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witloof chicory leaves. These observations suggest that *E. chrysanthemi* also produces a harpin. We report here the cloning, characterization, and mutagenesis of the *E. chrysanthemi* $hrpN_{Ech}$ gene and an investigation of the role of its product in plant interactions.

RESULTS

Molecular cloning of the *E. chrysanthemi* $hrpN_{Ech}$ gene.

We previously isolated 18 cosmids containing *E. chrysanthemi* DNA sequences hybridizing with the *E. amylovora hrp* cluster (Bauer *et al.* 1994). The pattern of restriction fragments released from these cosmids indicated they all contained overlapping inserts from the same region of the *E. chrysanthemi* genome (data not shown). The cosmids were probed in colony blots with a 1.3-kb *Hin*dIII fragment from pCPP1084, which contains the *E. amylovora hrpN* gene (Wei *et al.* 1992). pCPP2157, one f the three cosmids hybridizing with the probe, was digested with several restriction enzymes, and the location of the $hrpN_{Ech}$ gene in those fragments was determined by probing a Southern blot with the *E. amylovora* *Hin*dIII fragment. Two fragments, each containing the entire $hrpN_{Ech}$ gene, were subcloned into different vectors: pCPP2142 contained an 8.3-kb *Sal*I fragment in pUC119 (Vieira and Messing 1987), and pCPP2141 contained a 3.1-kb *Pst*I fragment in pBluescript II SK(–) (Stratagene, La J lla, CA).

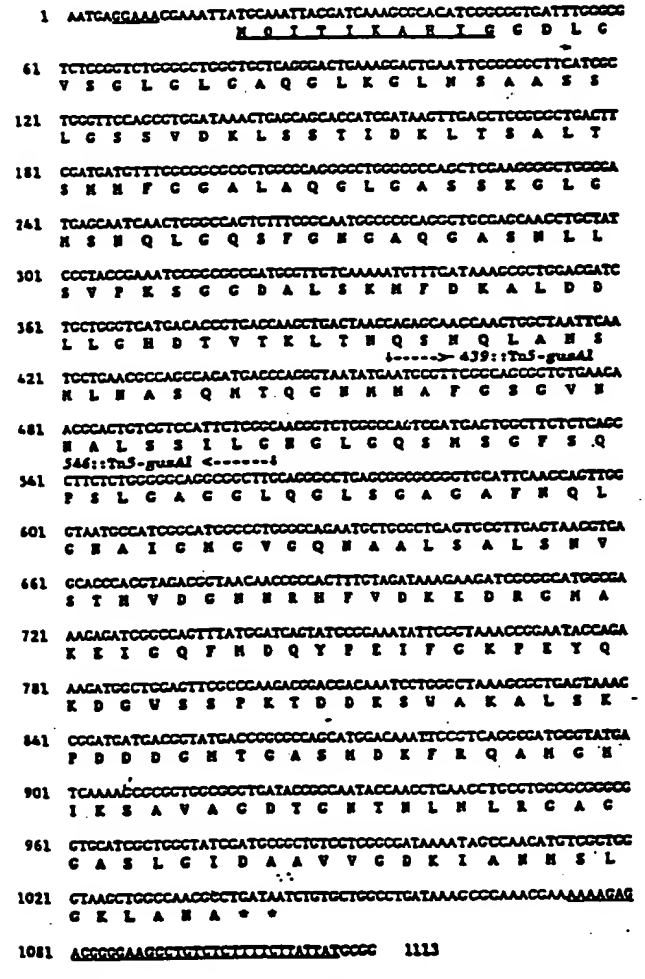

Fig. 1. DNA sequence of $hrpN_{Ech}$ and predicted amino acid sequence of its product. Underlined are the putative ribosome-binding site, the N-terminal amino acids confirmed by sequencing the product of pCPP2172, and a potential rho-independent transcription terminator. The location and orientation of two Tn*5-gusAI* insertions are also indicated and are numbered according to their location in the $hrpN_{Ech}$ open reading frame. The accession number for *hrpN* is L39897.

Sequence of $hrpN_{Ech}$.

The nucleotide sequence of a 2.4-kb region of pCPP2141 encompassing $hrpN_{Ech}$ was determined. The portion of that sequence extending from the putative ribosome-binding site through the $hrpN_{Ech}$ coding sequence to a putative rho-independent terminator is presented in Figure 1. The typical ribosome-binding site, consisting of GGAAA, was located eight bases upstream of the ATG translational initiation codon. A consensus *hrp* promoter sequence of $GGAACC(N)_{16}CACTCA$ (Bonas 1994) was found 97 bases upstream of the open reading frame (ORF), suggesting that $hrpN_{Ech}$ is a monocistronic operon. $hrpN_{Ech}$ codes for a predicted protein that has a molecular mass of 34.3 kDa, is rich in glycine (16.2%), and is lacking in cysteine. Comparison of the amino acid sequences of the predicted $hrpN_{Ea}$ and $hrpN_{Ech}$ products revealed extensive similarity, particularly in the C-terminal halves of the proteins (Fig. 2). The overall identity of the

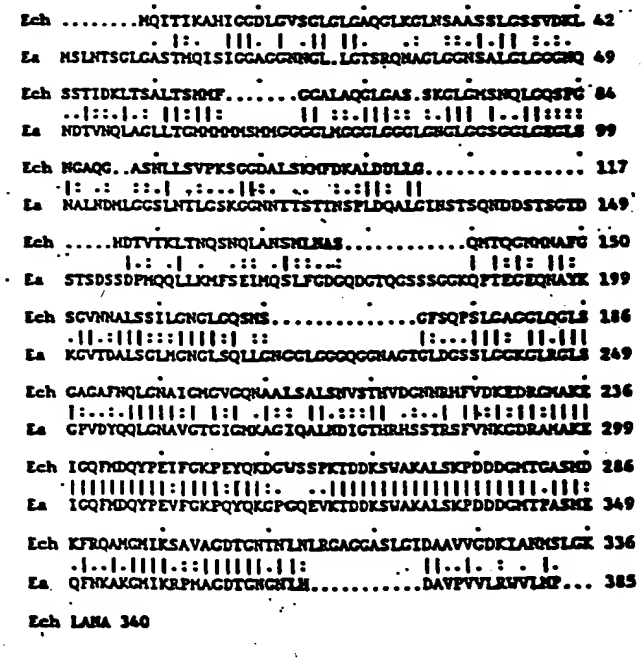

Fig. 2. Predicted amino acid sequences of the *hrpN* products $HrpN_{Ech}$ (Ech) of *Erwinia chrysanthemi* and $HrpN_{Ea}$ (Ea) of *E. amylovora*, aligned by the Gap program of the Genetics Computer Group Sequence Analysis Software Package (Devereaux *et al.* 1984). Two dots denote greater similarity than one dot.

hrpN genes and proteins was 66.9 and 45.5%, as determined by the FASTA and Gap algorithms, respectively (Devereaux *et al.* 1984; Pearson and Lipman 1988).

The direction of $hrpN_{Ech}$ transcription, the size of the predicted product, and the translation start site were confirmed by recloning the 3.1-kb *Pst*I fragment from pCPP2157 and selecting a clone with the fragment in pBluescript II SK(–) in the opposite orientation from pCPP2141, to produce pCPP2172. *E. coli* DH5α(pCPP2172) expressed $hrpN_{Ech}$ from the vector *lac* promoter and produced high levels of a protein with an estimated molecular mass of 36 kDa in sodium dodecyl sulfate (SDS) polyacrylamide gels, which is close to the predicted size (Fig. 3). Furthermore, the 10 N-terminal amino acids of the 36-kDa protein, determined by microsequencing following purification as described below, corresponded with the predicted N terminus of $HrpN_{Ech}$. As expected, no N-terminal signal sequence for targeting to the general export (Sec) pathway was discernible in the $HrpN_{Ech}$ sequence, and our data showed no evidence of processing of the N terminus.

Purification of the $hrpN_{Ech}$ product and demonstration of its HR elicitor activity in tobacco.

When DH5α(pCPP2172) cells were disrupted by sonication and then centrifuged, most of the $HrpN_{Ech}$ protein sedimented with the cell debris. However, soluble $HrpN_{Ech}$ could be released from this material by treatment with 4.5 M guanidine-HCl. This suggested that the protein formed inclusion bodies which could be exploited for purification. As detailed in Materials and Methods, we found that $HrpN_{Ech}$ reprecipitated when the guanidine-HCl was removed by dialysis against dilute buffer. The $HrpN_{Ech}$ precipitate could be washed and resuspended in buffer, in which it formed a fine suspension. SDS polyacrylamide gel analysis revealed the suspension to be electrophoretically homogeneous $HrpN_{Ech}$ (Fig. 3).

Cell-free lysates from *E. coli* DH5α(pCPP2172) cells grown in Luria-Bertani medium were infiltrated into tobacco leaves. Necrosis typical of the HR developed within 18 h, whereas leaf panels infiltrated with identically prepared lysates of DH5α(pBluescript SK–) showed no response (data not shown). The suspension of purified $HrpN_{Ech}$ at a concentration of 336 μg/ml also caused a necrotic response within 18 h that was indistinguishable from that caused by *E. chrysanthemi* CUCBP5030 or cell-free lysates from *E. coli* DH5α(pCPP2172) (Fig. 4). Tobacco plants vary in their sensitivity to harpins, and elicitation of the HR by $HrpN_{Ech}$ at lower concentrations was found to be variable. Consequently, a concentration of 336 μg/ml was used in all subsequent experiments. The concentration of $HrpN_{Ech}$ that is soluble in apoplastic fluids is unknown. To determine the heat stability of $HrpN_{Ech}$, the suspension of purified protein was incubated at 100° C for 15 min and then infiltrated into a tobacco leaf. There was no apparent diminution in its ability to elicit the HR (data not shown). These observations indicated that $HrpN_{Ech}$ is sufficient to account for the ability of *E. chrysanthemi* to elicit the HR in tobacco.

$hrpN_{Ech}$ mutants fail to elicit the HR in tobacco.

E. coli DH10B(pCPP2142) was mutagenized with Tn*5-gusA1* (Sharma and Signer 1990). Plasmid DNA was isolated

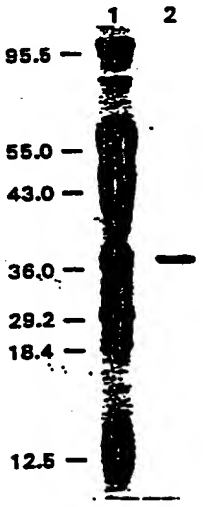

Fig. 3. Sodium dodecyl sulfate (SDS) polyacrylamide gel of purified $HrpN_{Ech}$. Purified $HrpN_{Ech}$ was solubilized in SDS loading buffer, electrophoresed through a 12% polyacrylamide gel, and stained with Coomassie Brilliant Blue. Lane 1, molecular weight markers (mid-range markers from Diversified Biotech, Boston, MA), with sizes in kilodaltons shown to the left; lane 2, $HrpN_{Ech}$.

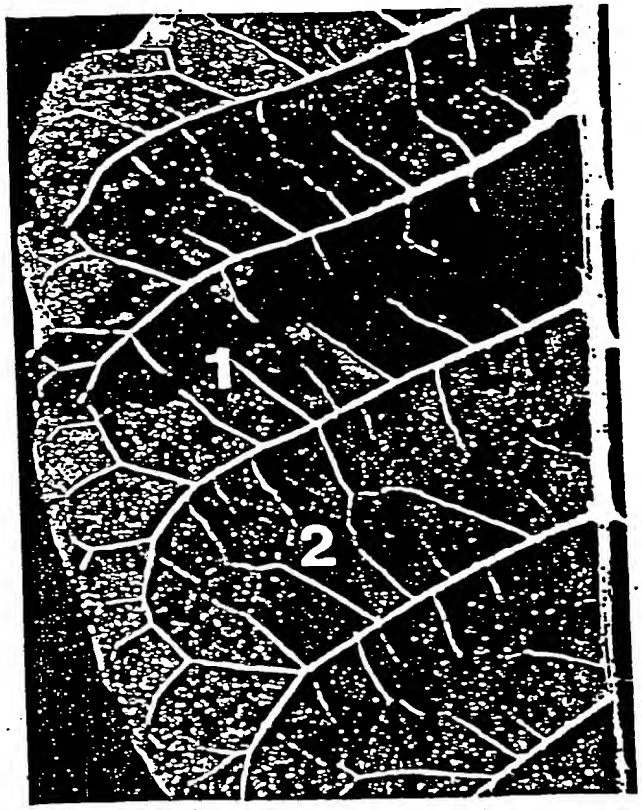

Fig. 4. Response of tobacco leaf tissue to purified $HrpN_{Ech}$. Leaf panel 1 was infiltrated with a suspension of purified $HrpN_{Ech}$ at a concentration of 336 μg/ml in 5 mM morpholinoethanesulfonic acid, pH 6.5. Panel 2 was infiltrated with buffer alone. The tissue in panel 1 collapsed 18 hr later. The leaf was photographed, 24 hr after infiltration, with a cross-polarized transilluminator, which enhances black and white visualization by making necrotic, desiccated areas that are typical of the hypersensitive response appear black.

from kanamycin-resistant colonies and transf rmed into *E. coli* DH5α, with selection for kanamycin resistance. Plasmids containing Tn*5-gusA1* were analyzed by restriction mapping. Two independent insertions in an 0.82-kb *Cla*I fragment internal to $hrpN_{Ech}$ were chosen for further study. The precise l cation and orientation of these insertions was determined by using a primer that hybridizes to Tn*5-gusA1* DNA upstream of *gusA* to sequence into the disrupted *E. chrysanthemi* DNA (Fig. 1). *E. coli* DH5α(pCPP2142) cells carrying the Tn*5-gusA1* insertion at nucleotide 439 of the $hrpN_{Ech}$ ORF (with *gusA* and $hrpN_{Ech}$ in the same orientation) produced dark blue colonies indicative of β-glucuronidase activity on LM agar (Hanahan 1983) supplemented with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (data not shown). Whether *gusA* was expressed from an *E. chrysanthemi* promoter or the vector *lac* promoter was not determined. The $hrpN_{Ech}439$::Tn*5-gusA1* and $hrpN_{Ech}546$::Tn*5-gusA1* mutations were marker-exchanged into the genome of *E. chrysanthemi* CUCPB5006 (Δ*pelABCE*) to produce mutants CUCPB5046 and CUCPB5045, respectively. Neither of the $hrpN_{Ech}$ mutants elicited a visible reaction in tobacco leaves (Fig. 5).

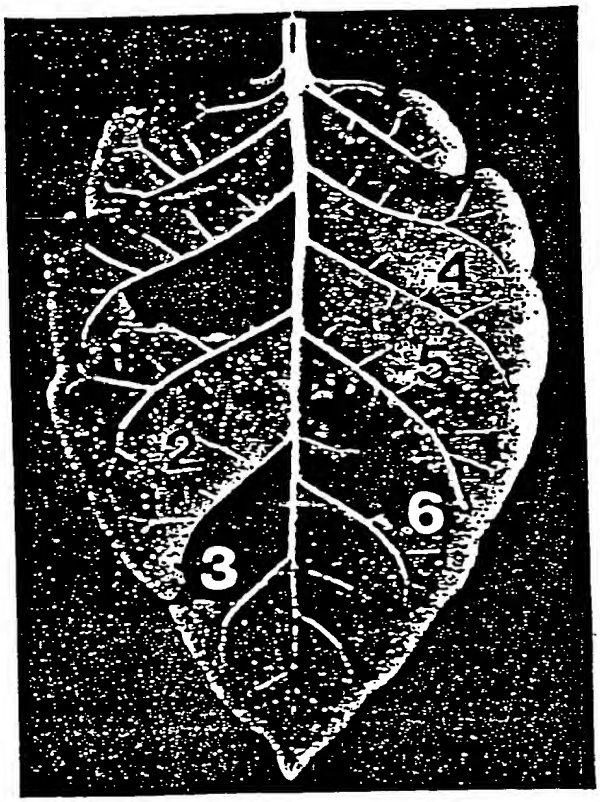

Fig. 5. Tobacco leaf showing that *Erwinia chrysanthemi hrpN* mutants do not elicit the hypersensitive response unless complemented with $hrpN^+$ pCPP2174. Bacteria were suspended at a concentration of 5×10^8 cells per milliliter in 5 mM morpholinoethanesulfonic acid, pH 6.5, and infiltrated into a tobacco leaf. The leaf was photographed 24 hr later under cross-polarized transillumination, as in Figure 4. 1, *E. chrysanthemi* CUCPB5006 (Δ*pelABCE*); 2, CUCPB5045 (Δ*pelABCE* $hrpN_{Ech}546$::Tn*5-gusA1* derivative of CUCPB5006); 3, CUCPB5045(pCPP2174); 4, buffer alone; 5, CUCPB5046 (Δ*pelABCE* $hrpN_{Ech}439$::Tn*5-gusA1* derivative of CUCPB5006); 6, CUCPB5046(pCPP2174).

E. chrysanthemi $hrpN_{Ech}$ mutations can be complemented *in trans* with $hrpN_{Ech}$ but n t with $hrpN_{Ea}$.

The presence of a typical rho-independent terminator just downstream of the $hrpN_{Ech}$ ORF suggested that mutations in the gene would not have polar effects on any other genes and that the HR elicitation phenotype should be restored by an $hrpN_{Ech}$ subclone. Because pCPP2172 carried 2 kb f *E. chrysanthemi* DNA in addition to $hrpN_{Ech}$, we constructed a precise subclone of the gene for this purpose. Oligonucleotides were used to amplify the $hrpN_{Ech}$ ORF by polymerase chain reaction and to introduce terminal *Nco*I and *Xho*I sites. The introduction of the restriction sites resulted in changing the second residue in the protein from glutamine to valine and adding a leucine and a glutamic acid residue to the C terminus. The resulting DNA fragment was ligated into *Xho*I- and *Nco*I-digested pSE280, creating pCPP2174, in which $hrpN_{Ech}$ was under control of the vector *tac* promoter. *E. chrysanthemi* CUCPB5045(pCPP2174) and CUCPB5046(pCPP2174) possessed HR elicitor activity (Fig. 5). HR elicitor activity could also be restored to these mutants by pCPP2142 and pCPP2172, but not by pCPP2141 (data not shown). Thus, the production of $HrpN_{Ech}$ is essential for elicitation of the HR by *E. chrysanthemi* CUCPB5006.

The feasibility of testing the interchangeability of the *hrpN* genes of *E. chrysanthemi* and *E. amylovora* was supported by the observation that HR elicitation activity could be restored to *hrpN* mutants in each species (*E. chrysanthemi* CUCPB5045 and *E. amylovora* Ea321T5) by their respective $hrpN^+$ subclones (pCPP2142 and pCPP1084). pCPP2142 was used for this purpose because preliminary immunoblot experiments indicated that the level of $hrpN_{Ech}$ expression by this plasmid, though relatively high, most closely approximated the expression of the native *hrpN* gene in *E. amylovora*. However, despite good heterologous expression of the *hrpN* genes, HR elicitation activity was not restored in either *E. amylovora* Ea321T5(pCPP2142) or *E. chrysanthemi*(pCPP1084) (data not shown). Thus, the genes do not appear to be functionally interchangeable.

E. chrysanthemi $hrpN_{Ech}$ mutants have a reduced ability to incite lesions in witloof chicory.

The $hrpN_{Ech}439$::Tn*5-gusA1* mutation was marker-exchanged into the genome of wild-type strain AC4150. The resulting mutant, CUCPB5049, was analyzed for its virulence in witloof chicory. Leaves were inoculated at small wounds with

Table 1. Effects of $hrpN_{Ech}$ mutation on the ability of *Erwinia chrysanthemi* to incite lesions on witloof chicory leaves

Strain	Number of lesions per 20 inoculations[a]	Size of lesions (mm^2, mean ± SD)[b]
AC4150 (wild type)	16	80 ± 55
CUCPB5049 ($hrpN_{Ech}429$::Tn*5-gusA1*)	8[c]	89 ± 42

[a] Each witloof chicory leaf was inoculated at two equivalent sites with 2×10^4 bacterial cells: one site received the $hrpN_{Ech}$ mutant, the other the parental wild-type strain; lesions were indicated by browning and maceration around the site of inoculation.

[b] Product of the length and width of the lesion.

[c] Different from the wild-type strain ($P < 0.05$), as determined by the McNemar test (Conover 1980).

2×10^4 cells of mutant and wild-type strains, as previously described (Bauer *et al.* 1994). The level of inoculum corresponded with the experimentally determined ED_{50} of the wild-type strain for the batch of chicory heads used. The approximate surface area of macerated lesions was determined 72 h after inoculation. These mutations did not abolish the pathogenicity of *E. chrysanthemi*, but they significantly reduced the number of successful lesions (Table 1). However, the $hrpN_{Ech}$ mutation had no significant effect on the size of the lesions produced in successful infections.

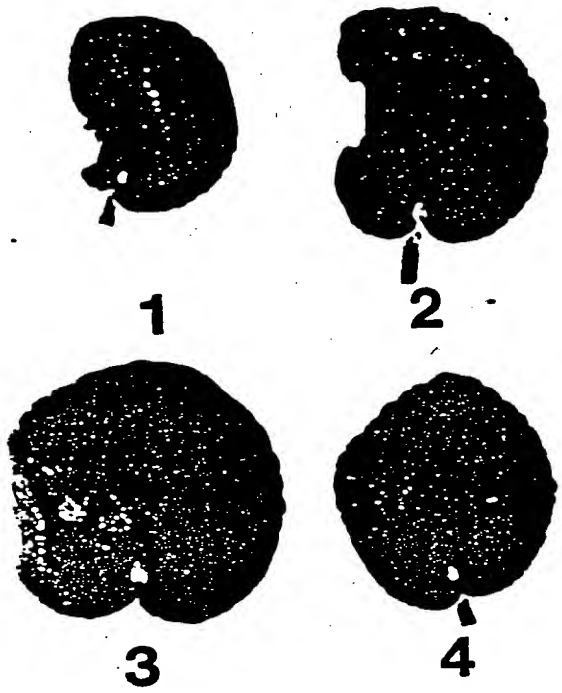

Fig. 6. African violet leaves showing rapid necrosis elicited by $HrpN_{Ech}$ and $HrpN^-_{Ech}$ Pel-deficient strains of *Erwinia chrysanthemi*. Leaves were inoculated with bacteria at a concentration of 3×10^8 cells per milliliter in 5 mM morpholinoethanesulfonic acid, pH 6.5, or purified $HrpN_{Ech}$ at a concentration of 336 μg/ml and photographed 24 hr later under cross-polarized transillumination, as in Figure 4. Buffer controls elicited no visible response (not shown). 1, *E. chrysanthemi* CUCPB5006 (Δ*pelABCE*); 2, CUCPB5030 (*outD*::Tn*phoA* derivative of CUCPB5006); 3, $HrpN_{Ech}$; 4, (left) CUCPB5045 (Δ*pelABCE* $hrpN_{Ech}546$::Tn*5-gusA1* derivative of CUCPB5006) and (right) CUCPB5063 (Δ*pelABCE* *outD*::Tn*phoA* $hrpN_{Ech}546$::Tn*5-gusA1* derivative of CUCPB5006).

Elicitation of a rapid necrosis in several plants by *E. chrysanthemi* is dependent on $HrpN_{Ech}$.

To determine whether *E. chrysanthemi* could cause an $HrpN_{Ech}$-dependent necrosis in plants other than tobacco, a variety of plants were infiltrated with purified $HrpN_{Ech}$ or inoculated with Pel-deficient *E. chrysanthemi* strains. The strains used were CUCPB5006; its $hrpN_{Ech}546$::Tn*5-gusA1* derivative, CUCPB5045; CUCPB5030 (Δ*pelABCE* *outD*::Tn*phoA*); and its $hrpN_{Ech}546$::Tn*5-gusA1* derivative, CUCPB5063. The results for African violet are shown in Figure 6, and results for all plants are summarized in Table 2. They yield several general observations. Plants responded either to both isolated $HrpN_{Ech}$ and $hrpN^-_{Ech}$ bacteria or to neither. Plants that responded to either treatment produced a nonmacerated, HR-like necrosis that developed between 12 and 24 h after infiltration. $hrpN_{Ech}$ mutants failed to elicit a response in any of the plants. The *out* mutation had no apparent influence on the responses elicited in the plants tested, indicating that residual Pel isozymes or other proteins traveling the Out pathway were not involved in producing the HR-like necrosis. The results argue that $HrpN_{Ech}$ is the only elicitor of the HR produced by *E. chrysanthemi*.

DISCUSSION

E. chrysanthemi was found to produce a protein with many similarities to the harpin of *E. amylovora*. The two proteins share significant amino acid sequence identity, similar physical properties, and the ability to elicit the HR in a variety of plants. Mutations in the $hrpN_{Ech}$ gene indicate that, as with *E. amylovora*, harpin production is required for elicitation of the HR. Furthermore, both harpins contribute to bacterial pathogenicity, albeit to different degrees. $HrpN_{Ea}$ is essential for *E. amylovora* to produce symptoms in highly susceptible, immature pear fruit (Wei *et al.* 1992), whereas $HrpN_{Ech}$ merely increases the frequency of successful *E. chrysanthemi* infections in susceptible witloof chicory leaves. Nevertheless, the finding that harpins play some role in the pathogenicity of

Table 2. Elicitation of necrosis in various plants by $HrpN_{Ech}$ and by *Erwinia chrysanthemi* strains variously deficient in Pel production and $HrpN_{Ech}$ production

Plant	$HrpN_{Ech}$[a]	CUCPB5006 (Δ*pelABCE*)[b]	CUCPB5045 (Δ*pelABCE* $hrpN_{Ech}546$::Tn*5-gusA1*)	CUCPB5030 (Δ*pelABCE* *outD*::Tn*phoA*)	CUCPB5063 (Δ*pelABCE* *outD*::Tn*phoA* $hrpN_{Ech}546$::Tn*5-gusA1*)
Tobacco	+	+	−	+	−
Tomato	+	+	−	+	−
Pepper	+	+	−	+	−
African violet	+	+	−	+	−
Petunia	+	+	−	+	−
Pelargonium	+	+	−	+	−
Squash	−	−	−	−	−
Zinnia	−	−	−	−	−

[a] Leaves on plants were infiltrated with $HrpN_{Ech}$ at a concentration of 336 μg/ml and observed macroscopically 24 hr later for necrosis and collapse of the infiltrated area (+) or absence of any response (−).

[b] Leaves on plants were infiltrated with bacteria at a concentration of 5×10^8/ml and scored for responses as described above.

such disparate pathogens suggests that these proteins have a conserved and widespread function in bacterial plant pathogenesis. We will consider below $HrpN_{Ech}$ with regard to the protein secretion pathways, extracellular virulence proteins, and wide host range of *E. chrysanthemi*.

E. chrysanthemi secretes proteins by multiple, independent pathways. Several protease isozymes are secreted by the Sec-independent (ABC-transporter, or Type I) pathway; pectic enzymes and cellulase are secreted by the Sec-dependent (general secretion, or Type II) pathway; and, $HrpN_{Ech}$ is likely to be secreted by the Sec-independent Hrp (Type III) pathway (Salmond 1994). The expectation that $HrpN_{Ech}$ is secreted by the Hrp pathway is supported by several lines of indirect evidence: (i) Hrp secretion pathway mutants have revealed that other members of this class of glycine-rich, heat-stable elicitor proteins—the *E. amylovora* $HrpN_{Ea}$, *P. syringae* pv. *syringae* HrpZ, and *P. solanacearum* PopA1 proteins—are secreted by this pathway (He *et al.* 1993; Wei and Beer 1993; Arlat *et al.* 1994); (ii) mutation of the *E. chrysanthemi* homolog of an *E. amylovora* gene involved in $HrpN_{Ea}$ secretion abolishes the ability of *E. chrysanthemi* to elicit the HR, whereas mutation of the Out (Type II) pathway of *E. chrysanthemi* does not abolish the HR; and (iii) $HrpN_{Ech}$ appears to be the only HR elicitor produced by *E. chrysanthemi* (as discussed further below), suggesting that the effect of the putative *hrp* secretion gene mutation is on $HrpN_{Ech}$. Our attempts to directly demonstrate *hrp*-dependent secretion of $HrpN_{Ech}$ have been thwarted by the apparent instability of the protein in *E. chrysanthemi*. Using the cell fractionation and immunoblotting procedures of He *et al.* (1993) and polyclonal anti-$HrpN_{Ea}$ antibodies that cross-react with $HrpN_{Ech}$ (Wei *et al.* 1992), we have observed the presence of $HrpN_{Ech}$ in the cell-bound fraction of *E. chrysanthemi* (D. W. Bauer, unpublished). However, some culture preparations unexpectedly lack the protein, and no preparations reveal accumulation of the protein in the culture supernatant fraction. It is possible that $HrpN_{Ech}$ aggregates upon secretion and therefore precipitates from the medium. It is interesting that several of the *Yersinia* spp. Yop virulence proteins aggregate in the medium upon secretion via the Type III pathway (Michiels *et al.* 1990). Similarly, $HrpN_{Ea}$ has a propensity to form aggregates or to associate with an insoluble membrane fraction (Wei *et al.* 1992).

It is significant that there is little difference in the plant interaction phenotypes of *E. chrysanthemi* mutants deficient in either $HrpN_{Ech}$ or a putative component of the Hrp secretion pathway (Bauer *et al.* 1994). Both mutations abolish the ability of Pel-deficient strains to elicit the HR, and they both reduce the frequency of successful infections incited by fully pectolytic strains in witloof chicory leaves without affecting

Table 3. Bacterial strains and plasmids used in this study

Designation	Relevant characteristic[a]	Reference or source
Escherichia coli		
ED8767	*supE44 supF58 hsdS3*($r_B^- m_B^-$) *recA56 galK2 galT22 metB1*	Sambrook *et al.* 1989
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1* Nal^r	Hanahan 1983
DH10B	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80 *lacZ*ΔM15 Δ*lacX74 deoR recA1 endA1 araD*139 Δ(*ara, leu*)7697 *galU galK rpsL nupG*	Life Technologies, Grant *et al.* 1990
Erwinia chrysanthemi		Life Technologies, Inc.
EC16	Wild-type strain	Burkholder *et al.* 1953
AC4150	Spontaneous Nal^r derivative of EC16	Chatterjee *et al.* 1983
CUCPB5006	Δ(*pelB pelC*)::28bp Δ(*pelA pelE*) derivative of AC4150	He and Collmer 1990
CUCPB5030	*outD*::Tn*phoA* derivative of CUCPB5006	Bauer *et al.* 1994
CUCPB5045	$hrpN_{Ech}$*546*::Tn*5-gusA1* derivative of CUCPB5006	This work
CUCPB5046	$hrpN_{Ech}$*439*::Tn*5-gusA1* derivative of CUCPB5006	This work
CUCPB5063	$hrpN_{Ech}$*546*::Tn*5-gusA1* derivative of CUCPB5030	This work
CUCPB5049	$hrpN_{Ech}$*439*::Tn*5-gusA1* derivative of AC4150	This work
Erwinia amylovora		
Ea321	Wild type	ATCC 49947
Ea321T5	$hrpN_{Ea}$::Tn*5tac1* derivative of Ea321	Wei *et al.* 1992
Plasmids and phage		
pBluescript II SK(−)	Amp^r	Stratagene, La Jolla, CA
pCPP19	Cosmid vector, Sp^r/Sm^r	D. W. Bauer
pUC119	Amp^r plasmid vector	Vieira and Messing 1987
pSE280	Amp^r plasmid vector with superpolylinker downstream of tac promoter	Brosius 1989
pCPP2030	pCPP19 carrying *E. chrysanthemi* DNA hybridizing with *E. amylovora hrp* genes in pCPP1033	Bauer *et al.* 1994
pCPP1084	pBluescript M13+ carrying $hrpN_{Ech}$ on 1.3-kb *Hin*dIII fragment	Wei *et al.* 1992
pCPP2157	pCPP19 carrying *E. chrysanthemi* DNA hybridizing with *E. amylovora hrpN*	This work
pCPP2142	8.3-kb *Sal*I subclone from pCPP2157 in pUC119	This work
pCPP2141	3.1-kb *Pst*I subclone from pCPP2157 in pBluescript II SK(−) $hrpN_{Ech}$ in the orientation opposite that of the vector *lac* promoter	This work
pCPP2172	3.1-kb *Pst*I subclone from pCPP2157 in pBluescript II SK(−) $hrpN_{Ech}$ in same orientation as vector *lac* promoter	This work
pCPP2174	1.0-kb $hrpN^+_{Ech}$ polymerase chain reaction product cloned in *Nco*I-*Hin*dIII sites of pSE280	This work
λ::Tn*5-gusA1*	Tn*5* derivative for generating transcriptional fusions with *uidA* reporter; Kan^r, Tet^r	Sharma and Signer 1990

[a] Amp^r = ampicillin resistance; Kan^r = kanamycin resistance; Nal^r = nalidixic acid resistance; Sm^r = streptomycin resistance; Sp^r = spectinomycin resistance; Tet^r = tetracycline resistance.

the size of the macerated lesions that do develop. This pattern contrasts with that observed in mutations affecting Pel isozymes and the Out pathway. Virulence, as measured by maceration, is merely reduced by individual *pel* mutations, whereas it is abolished by *out* mutations. This is because multiple Pel isozymes (and possibly other enzymes) contribute quantitatively to virulence, but all of the Pel isozymes appear to be dependent on the Out pathway for secretion from the bacterial cell. The simplest interpretation of the observations with *E. chrysanthemi hrp* mutants is that $HrpN_{Ech}$ is the only protein traveling the Hrp pathway that has a detectable effect on the interaction of *E. chrysanthemi* EC16 with the plants tested.

The primacy of $HrpN_{Ech}$ in the *E. chrysanthemi* Hrp system is further supported by the observations that $hrpN_{Ech}$ mutants failed to elicit necrosis in any of the several plants tested and that all plants responding with apparent hypersensitivity to $HrpN^{+}_{Ech}$ strains also responded to isolated $HrpN_{Ech}$. Several of the plants sensitive to $HrpN_{Ech}$ are also susceptible to bacterial soft rots. This is particularly significant for African violet, whose interactions with *E. chrysanthemi* have been extensively studied (Barras *et al.* 1994). Thus, $HrpN_{Ech}$ elicits HR-like responses in plants that are susceptible to *E. chrysanthemi* infections under appropriate environmental conditions. The significance of this for the wide host range of the bacterium requires further investigation, and virulence tests with $hrpN_{Ech}$ mutants and additional susceptible plants are needed to determine the general importance of $HrpN_{Ech}$ and the Hrp system in *E. chrysanthemi*. For example, our present data do not address the possibility that other proteins secreted by the Hrp pathway, which are not elicitors of the HR in the plants we tested, may contribute to pathogenesis in hosts other than witloof chicory.

An important question is whether bacteria expressing heterologous harpins will be altered in pathogenicity. The *hrpN* genes of *E. chrysanthemi* and *E. amylovora* are particularly attractive for experiments addressing this because of the similarity of the harpins and the dissimilarity of the diseases produced by these bacteria. Unfortunately, attempts to restore the HR phenotype to *E. chrysanthemi* and *E. amylovora hrpN* mutants with heterologous $hrpN^{+}$ subclones failed. Since the *hrpN* genes in each subclone successfully complemented *hrpN* mutations in homologous bacteria and were expressed in heterologous bacteria, the problem is most likely the secretion of the harpins by heterologous Hrp systems. A similar problem has been encountered with heterologous secretion of Pel and cellulase via the Out pathway in *E. chrysanthemi* and *E. carotovora*, species that are more closely related to each other in this rather heterogeneous genus than *E. chrysanthemi* and *E. amylovora* are (He *et al.* 1991; Py *et al.* 1991).

In conclusion, two classes of proteins contribute to the pathogenicity of *E. chrysanthemi*—a single harpin and a battery of plant cell wall–degrading pectic enzymes. The observation that such a highly pectolytic organism also produces a harpin suggests the fundamental importance of harpins in the pathogenicity of gram-negative bacteria. The observation that an $hrpN_{Ech}$::Tn*5-gusA1* mutation reduced the ability of a fully pectolytic strain of *E. chrysanthemi* to initiate lesions in susceptible chicory leaves, but did not reduce the size of lesions that did develop, suggests that $HrpN_{Ech}$ contributes specifically to an early stage of pathogenesis. An attractive possibility is that $HrpN_{Ech}$ releases nutrients to the apoplast for bacterial nutrition before the *pel* genes are fully expressed (Collmer and Bauer 1994). Patterns of *pel* and $hrpN_{Ech}$ expression *in planta* will likely yield further clues to the role of the *E. chrysanthemi* harpin in soft-rot pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

Bacterial stains and plasmids are listed in Table 3. *E. chrysanthemi* was routinely grown in King's medium B (King *et al.* 1954) at 30° C, *E. coli* in LM medium (Hanahan 1983) at 37° C, and *E. amylovora* in Luria-Bertani medium at 28–30° C. The following antibiotics were used in selective media in the amounts indicated (in μg/ml), except where noted: ampicillin (100), kanamycin (50), spectinomycin (50), and streptomycin (25).

General DNA manipulations.

Plasmid DNA manipulations, colony blotting, and Southern blot analyses were performed by standard techniques (Sambrook *et al.* 1989). Deletions for sequencing were constructed with the Erase-a-Base kit (Promega, Madison, WI). Double-stranded DNA sequencing templates were prepared with Qiagen Plasmid Mini Kits (Chatsworth, CA). Sequencing was performed with the Sequenase Version 2 kit (U.S. Biochemical, Cleveland, OH). The Tn*5-gusA1* insertion points were determined on an Automated DNA Sequencer (model 373A, Applied Biosystems, Foster City, CA) by the Cornell Biotechnology Center. DNA sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (Devereaux *et al.* 1984). Comparison of $HrpN_{Ech}$ and $HrpN_{Ea}$ by the Gap program was done with a gap weight of 5.0 and a gap length weight of 0.3. Marker exchange mutagenesis was performed as previously described (Bauer *et al.* 1994). The oligonucleotide used to determine the location of Tn*5-gusA1* insertions in $hrpN_{Ech}$ was TGACCTGCAGCCAAGCTTTCC. The oligonucleotide used as the first primer to amplify the $hrpN_{Ech}$ ORF and introduce an *Nco*I site at the 5′ end of the gene was AGTACCATGGTTATTACGATCAAAGCGCAC; the one used as the second primer to introduce an *Xho*I site at the 3′ end of the gene was AGATCTCGAGGGCGTTGGCCAGCTTACC. Primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Protein manipulations.

$HrpN_{Ech}$ was purified from *E. coli* DH5α(pCPP2172) cultures grown at 30° C to stationary phase in 50 ml of Terrific Broth (Sambrook *et al.* 1989) supplemented with ampicillin at a concentration of 200 μg/ml. Cells were lysed by lysozyme treatment and sonication as previously described (Sambrook *et al.* 1989). The lysate pellet was washed twice with 9 vol of lysis buffer containing 0.5% Triton X-100 and 10 mM EDTA, pH 8.0, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF); the lysate was reharvested each time by centrifugation at 12,000 × *g* for 15 min. The pellet was resuspended in 2.0 ml of lysis buffer containing 0.1 mM PMSF, dissolved by the addition of 2.5 ml of 8 M guanidine-HCl in lysis buffer, and then diluted with 5.0 ml of water. The protein solution was dialyzed in SpectraPor #1 dialysis tubing against 2.0 liters of 5 mM morpholinoethanesulfonic acid (MES), pH 6.5, containing 0.05 mM PMSF. The precipitate that formed dur-

ing dialysis and the solution were centrifuged for 15 min at 4,300 × *g*. The pellet was washed once with 10 ml of a solution containing 5 mM MES, pH 6.5, and 0.1 mM PMSF and then resuspended in 2.0 ml of the same buffer. Protein concentrations of hom geneous suspensions were determined following dissolution in the reagents of the dye-binding assay of Bradford (1976). Proteins in crude cell lysates or following purification were resolved by electrophoresis through an SDS 12% polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue R. The N-terminal sequence of purified $HrpN_{Ea4}$ was determined at the Cornell University Biotechnology Program Protein Analysis Facility.

Plant assays.

For HR assays, tobacco (*Nicotiana tabacum* L. cv. Xanthi), tomato (*Lycopersicon esculentum* Mill. cv. Sweet 199), pepper (*Capsicum annuum* L. cv. Sweet Hungarian), African violet (*Saintpaulia ionantha* H. Wendl. cv. Paris), petunia (*Petunia grandiflora* Juss. cv. Blue Frost), pelargonium (*Pelargonium hortorum* Bailey), winter squash (*Cucurbita maxima* Duchesne), and zinnia (*Zinnia elegans* Jacq.) plants were grown under greenhouse conditions or purchased at a local garden shop and then maintained in the laboratory at room temperature, with incident daylight supplemented with a 500-W halogen lamp. Witloof chicory (*Cichorium intybus* L.) was purchased as "Belgian endive" heads from a local supermarket. Bacterial inoculum was prepared and delivered as previously described (Bauer *et al.* 1994). Briefly, to assay soft-rot pathogenesis, 5 µl of inoculum was applied to a small wound in detached chicory leaves; to assay for HR elicitation, inoculum was infiltrated with a needle-less plastic syringe into leaves on plants.

ACKNOWLEDGMENTS

We thank Kent Loeffler for photography. This work was supported by NRI Competitive Grants Program/USDA grants 91-37303-6321 (AC), 94-37303-0734 (AC), and 91-37303-6430 (SVB).

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